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Physiologic cyclic stretch inhibits apoptosis in vascular endothelium

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Abstract **Since apoptosis of endothelial cells (ECs) plays an important role in the pathogenesis of atherosclerosis, we investigated the effect of cyclic stretch on EC apoptosis. Application of moderate, physiologic levels of cyclic stretch (6–10% at 1 Hz) inhibited EC apoptosis. This anti-apoptotic effect was dependent on the activation of phosphatidylinositol 3-kinase and associated with the activation of Akt and the phosphorylation of Bad. Interestingly, a higher potentially pathologic level of cyclic stretch (20% at 1 Hz) stimulated EC apoptosis. The ability of physiologic cyclic stretch to inhibit EC apoptosis may provide a previously unrecognized mechanism by which hemodynamic forces exert an anti-atherogenic effect.**

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Key words: Cyclic stretch; Endothelial cell; Apoptosis

1. Introduction

Emerging evidence suggests that apoptosis of endothelial cells (ECs) plays a pivotal role in the development of atherosclerosis. Many of the classical pro-atherogenic factors, including oxidized low density lipoprotein, inflammatory cytokines, and reactive oxygen species, are potent inducers of EC apoptosis whereas known atheroprotective factors such as NO and high density lipoprotein inhibit EC apoptosis [1–4]. Recently, apoptotic ECs have been directly detected in human coronary atherosclerotic plaques [5]. Interestingly, EC apoptosis and atherosclerotic lesions preferentially develop in regions of disturbed blood flow suggesting an important link between local hemodynamic forces and the pathobiological processes leading to EC death and atherosclerosis [5,6]. In support of this hypothesis, laminar fluid shear stress has been shown to be a potent inhibitor of EC apoptosis [7–9].

In addition to fluid shear stress, vascular ECs are continuously subjected to cyclic stretch (or strain), which arises from the periodic change in vessel diameter as a result of pulsatile blood flow. Recent studies indicate that cyclic stretch exerts significant effects on the vascular endothelium. The imposition of cyclic mechanical stretch alters EC orientation and may play a fundamental role in regulating vascular tone and blood fluidity by stimulating the release of humoral factors from the endothelium [10,11]. In the present study, we investigated whether cyclic stretch also regulates EC death.

2. Materials and methods

2.1. Materials

Wortmannin and LY-294002 were from Alexis Biochemicals; RNase A and proteinase K were from Boehringer Mannheim; recombinant human tumor necrosis factor-α (TNFα) was from Genzyme; rabbit polyclonal Akt and sheep polyclonal phospho-Akt (Ser-473) were from Upstate Biotechnology; phospho-Bad (Ser-136) antibody was from New England Biolabs; goat polyclonal β-actin antibody was from Santa Cruz Biotechnology; all other reagents were from Sigma.

2.2. Cell culture

Bovine aortic ECs were purchased from VEC Technologies and grown in Dulbecco's modified Eagle's medium supplemented with 10% serum, 5 mM TES, and 5 mM HEPES. Human aortic ECs were purchased from Clonetics Corporation and grown in M199 medium containing 20% serum, 50 μg/ml endothelial cell growth supplement, and 50 μg/ml heparin. Culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) and ECs propagated in 5% CO_2 at 37°C.

2.3. Application of cyclic strain

ECs were plated onto 6-well Bioflex plates coated with type I collagen (Flexercell Corporation). The thickness of the silicon elastomer on the bottom of the plate varies along the diameter of the plate such that a near homogeneous strain profile is obtained throughout the membrane. Cells were subjected to mechanical deformation of 6–20% at 1 Hz using the Flexercell Strain Unit (FX 3000, Flexercell Corporation), as previously described [10].

2.4. Cell apoptosis and viability

Apoptosis was monitored by measuring DNA fragmentation and the activation of caspase-3 [12]. DNA fragmentation was examined by agarose gel electrophoresis while caspase-3 activity was determined using a colorimetric assay which monitors the cleavage of the *p*-nitroanilide-conjugated caspase-3 substrate DEVD (Clontech). ECs (1×10^6 cells) were trypsinized, washed in ice-cold phosphate-buffered saline (PBS), and suspended in lysis buffer (50 mM HEPES pH 7.5, 10% sucrose, 0.1% Triton X-100) on ice. Following centrifugation at $14\,000\times g$ for 5 min at 4°C, supernatants were incubated with 50 μM of DEVD and absorbance measured at 405 nm. Cell viability was assessed by measuring the uptake of the membrane-impermeable stain trypan blue, as we have previously described [12].

2.5. Western blotting

Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blots electrophoretically transferred to nitrocellulose membranes and blocked in PBS and non-fat milk (5%). Blots were incubated with antibodies directed against phosphorylated Akt (1:1000 dilution), Akt (1:1000 dilution), phospho-Bad (1:500 dilution), or the housekeeping protein β-actin (1:100 dilution). Membranes were then washed in PBS and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat,

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Abbreviations: ECs, endothelial cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNFα, tumor necrosis factor-α; PI3K, phosphatidylinositol 3-kinase

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or rabbit anti-sheep antibody. After further washing with PBS, blots were incubated in commercial chemoluminescence reagents (Amersham).

2.6. Mitochondrial cytochrome c release

EC lysates were fractionated, proteins (50 μg) resolved by SDS–PAGE, and cytochrome *c* analyzed by Western blotting, as we have previously described [12], using a monoclonal antibody (5 μg/ml) (R&D Systems) that specifically recognizes the denatured form of cytochrome *c*.

2.7. Statistics

Results are expressed as the means ± S.E.M. Statistical analysis was performed with a Student's two-tailed *t*-test or ANOVA when more than two treatments were compared. *P* values less than 0.05 were considered to be statistically significant.

3. Results

Treatment of bovine aortic ECs with TNFα (40 ng/ml) for 48 h resulted in a marked decrease in the number of viable cells (Fig. 1A). This cytotoxic effect of TNFα was associated with an increase in EC apoptosis, as reflected by a pronounced DNA ladder (Fig. 1B) and a significant elevation in caspase-3 activity (Fig. 1C). However, the application of cyclic stretch (6% at 1 Hz) reversed the apoptotic effect of TNFα on bovine aortic ECs. Cyclic stretch increased the number of viable cells following cytokine treatment and blocked cytokine-mediated DNA laddering and caspase-3 activation (Fig. 1A–C). In addition, Western blot analysis revealed that cytochrome *c* was found predominantly in the membrane fraction in control ECs (Fig. 1D). As previously reported [4], minor amounts of cytochrome *c* were also detected in the cytosolic fraction of control ECs (Fig. 1D). This likely represents leakage of cytochrome *c* from the mitochondria during the fractionation procedure. However, following TNFα treatment, substantial amounts of cytochrome *c* were detected in the cytosolic fraction (Fig. 1D). This TNFα-mediated translocation of cytochrome *c* from the membrane to the cytosolic fraction was blocked by 6% cyclic stretch (Fig. 1D). Exposure of bovine aortic EC to cyclic mechanical stretch also inhibited DNA fragmentation following serum deprivation (Fig. 2A). Similarly, application of cyclic stretch (6%) blocked DNA laddering secondary to serum depletion in human aortic ECs (Fig. 2B).

Interestingly, the cytoprotective action of cyclic strain was dependent on the degree of strain. While 6 and 10% strain protected bovine aortic ECs against TNFα-mediated cytotoxicity, the application of 20% stretch failed to protect ECs (Fig. 3A). In fact, 20% cyclic stretch induced DNA laddering in control ECs and potentiated TNFα-mediated DNA laddering (Fig. 3B).

We next examined the molecular mechanism by which cyclic strain inhibits EC apoptosis. Since cyclic stretch stimulates the synthesis of NO [10], a potent inhibitor of EC apoptosis [3], we examined whether NO was a mediator of the anti-apoptotic effect of cyclic stretch. However, inhibition of NO synthase with methyl-L-arginine (1 mM) failed to reverse the inhibitory effect of cyclic stretch (6%) on DNA laddering (Fig. 4A). In contrast, the phosphatidylinositol 3-kinase (PI3K) in-

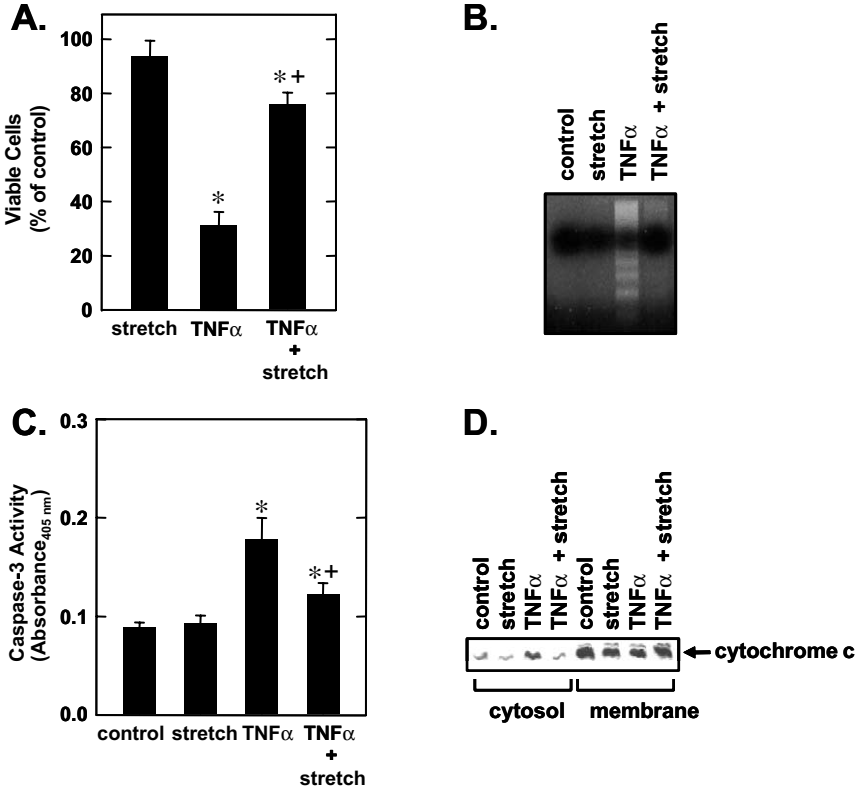

Fig. 1. Cyclic stretch (6%) inhibits TNFα (40 ng/ml for 48 h)-mediated apoptosis in bovine aortic ECs. A: Effect of cyclic stretch on cell viability. Results are means ± S.E.M. of four separate experiments. B: Effect of cyclic stretch on DNA laddering. Data are representative of four separate experiments. C: Effect of cyclic stretch on caspase-3 activity. Results are means ± S.E.M. of three separate experiments. D: Effect of cyclic stretch on the release of mitochondrial cytochrome *c*. Data are representative of three separate experiments. *Statistically significant effect of TNFα. +Statistically significant effect of cyclic stretch.

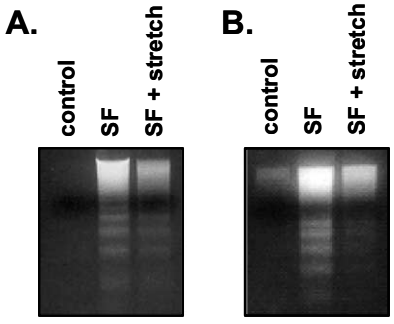

Fig. 2. Cyclic stretch (6%) inhibits apoptosis in bovine (A) and human (B) aortic ECs following 48 h of serum deprivation. Data are representative of three separate experiments.

hibitors, wortmannin (100 nM) and LY-294002 (10 μM), reversed the anti-apoptotic effect of cyclic stretch (6%) (Fig. 4B,C). These PI3K inhibitors had no effect on TNFα-stimulated apoptosis (Fig. 4B,C). Application of cyclic strain (6%) to ECs also induced the activation of protein kinase, Akt, which is a downstream target of PI3K [13]. Akt activity was measured by Western blotting with a phosphospecific Akt antibody, which has been shown to correlate with enzyme activity [14]. Exposure of vascular ECs to cyclic strain (6%) stimulated the phosphorylation of Akt (Fig. 5A) and this was not affected by TNFα (data not shown). Since Akt has been reported to phosphorylate and inactivate the pro-apoptotic Bcl2 family member, Bad, we investigated the effect of cyclic stretch on Bad phosphorylation [15]. Exposure of ECs to cyclic strain (6%) induced the phosphorylation of Bad, as detected by Western blotting with a phosphospecific antibody against the Akt phosphorylation site (Ser-136), and this was blocked by the PI3K inhibitors wortmannin (100 nM) and LY-294002 (10 μM) (Fig. 5B).

4. Discussion

In the present study, we are the first to demonstrate that physiologic levels of cyclic stretch (6–10%) inhibit apoptosis in arterial ECs. The anti-apoptotic effect of these moderate levels of cyclic stretch is dependent on the activation of PI3K and is associated with the activation of Akt and the phosphorylation of Bad. In contrast, a high level of cyclic stretch (20%) actually induces programmed cell death. These findings establish cyclic stretch as a novel regulator of EC death.

Treatment of vascular ECs with TNFα stimulates apoptosis, as demonstrated by a decrease in cell viability, DNA laddering, and caspase-3 activation. However, the simultaneous application of cyclic stretch (6 and 10%) to vascular ECs exerts a significant inhibitory effect on cytokine-mediated apoptosis. Similarly, cyclic stretch (6%) blocks apoptosis induced by serum deprivation in both bovine and human aortic ECs, suggesting that physiologic cyclic stretch functions as a general inhibitor of vascular EC apoptosis. The levels of cyclic stretch required to inhibit apoptosis are comparable to those reported in large peripheral vessels under normotensive conditions and thus represent physiologically relevant levels [15].

The mechanism by which cyclic stretch inhibits apoptosis in ECs involves the activation of PI3K. We found that cyclic stretch stimulates the phosphorylation and activation of Akt, which is a downstream target of PI3K. Moreover, two structurally dissimilar pharmacological inhibitors of PI3K, wortmannin and LY-294002, are able to reverse the anti-apoptotic effect. Akt is a known inhibitor of apoptosis that blocks programmed cell death by phosphorylating specific downstream targets. Akt stimulates the phosphorylation and activation of endothelial NO synthase [16,17], and NO promotes EC survival by inhibiting caspases via nitrosylation [13]. However, NO does not mediate the anti-apoptotic effect of cyclic stretch since the NO synthase inhibitor, methyl-L-arginine, fails to reverse the apoptosis-suppressive action of cyclic stretch. Recently, Akt has been shown to stimulate the phosphorylation and inactivation of the pro-apoptotic protein Bad [13]. In its unphosphorylated form, Bad induces apoptosis by heterodimerization with other Bcl-2 family members, leading to the release of cytochrome *c* from the mitochondria. We found that cyclic stretch stimulates the phosphorylation of Bad at Ser-136, in a PI3K-dependent manner. The phosphorylation of Bad results in its association with cytoplasmic 14-3-3 adapter proteins, thereby sequestering Bad from its targets at the mitochondria [13]. The phosphorylation of Bad by cy-

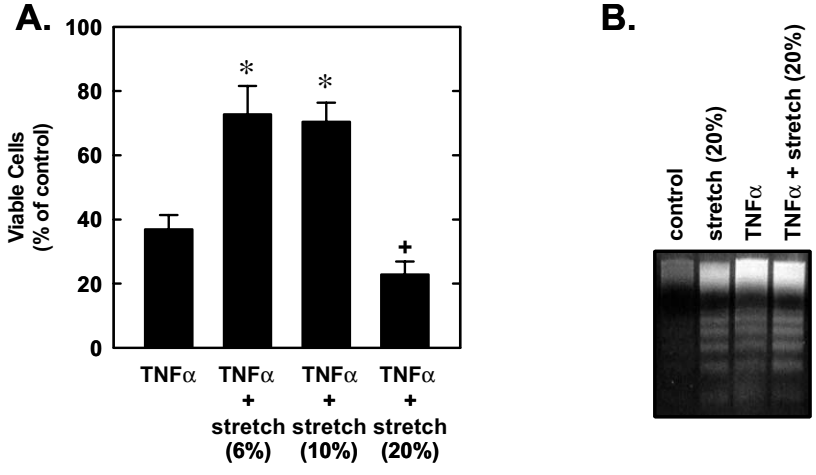

Fig. 3. Anti-apoptotic effect of cyclic stretch is dependent on the degree of cyclic stretch. A: Effect of cyclic stretch (6, 10, or 20%) on TNFα (40 ng/ml for 48 h)-mediated cytotoxicity. Results are means ± S.E.M. of four separate experiments. *Statistically significant effect of TNFα. +Statistically significant effect of cyclic stretch. B: Effect of cyclic stretch (20%) on TNFα (40 ng/ml for 48 h)-mediated DNA laddering. Data are representative of three separate experiments.

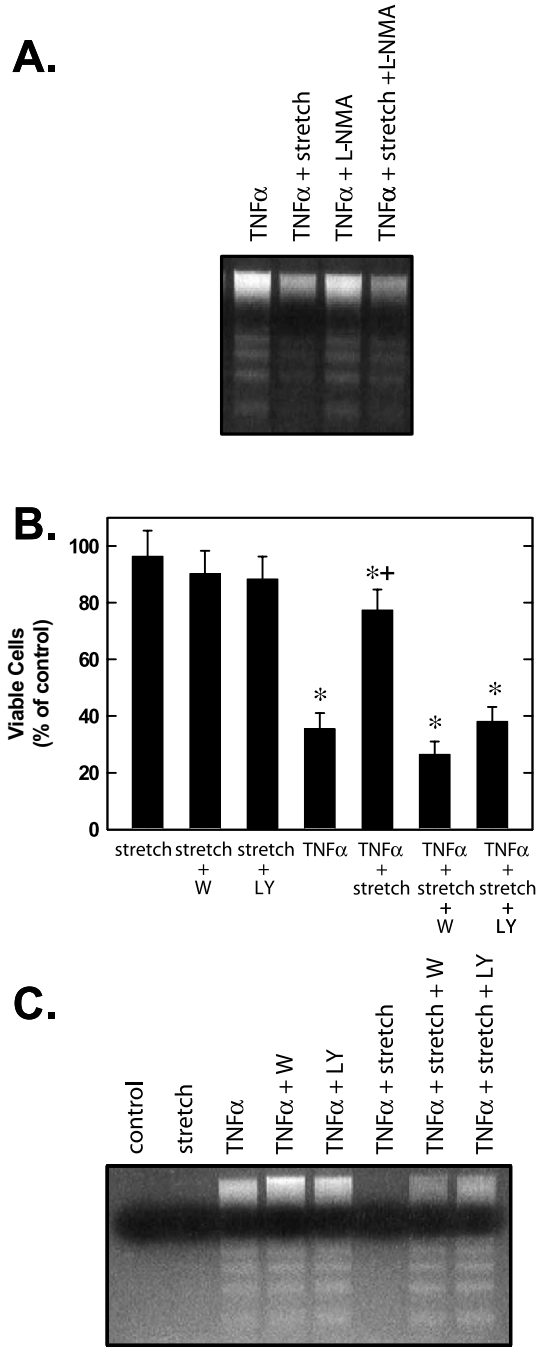

Fig. 4. Role of NO and PI3K in the anti-apoptotic effect of cyclic stretch (6%). A: Effect of methyl-L-arginine (L-NMA; 1 mM) on TNFα (40 ng/ml for 48 h)-mediated DNA laddering in the presence and absence of cyclic stretch. Data are representative of three separate experiments. B: Effect of the PI3K inhibitors, wortmannin (W; 100 nM) or LY-294002 (LY; 10 μM), on TNFα (40 ng/ml for 48 h)-mediated cytotoxicity in the presence and absence of cyclic stretch. Results are means ± S.E.M. of three separate experiments. *Statistically significant effect of TNFα. +Statistically significant effect of stretch. C: Effect of the PI3K inhibitors, wortmannin (W; 100 nM) or LY-294002 (LY; 10 μM), on TNFα (40 ng/ml for 48 h)-mediated DNA laddering. Data are representative of three separate experiments.

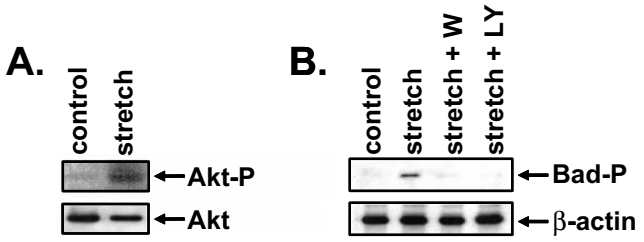

Fig. 5. Cyclic stretch stimulates the phosphorylation of Akt (Akt-P) and Bad (Bad-P) in bovine aortic ECs. A: Effect of cyclic stretch (6% for 30 min) on Akt-P. B: Effect of cyclic stretch (6% for 30 min) on Bad-P in the presence and absence of wortmannin (W; 100 nM) and LY-294002 (LY; 10 μM).

clic stretch likely contributes to its ability to block mitochondrial cytochrome *c* release. In addition to directly phosphorylating Akt and Bad, cyclic stretch may further promote the phosphorylation of these proteins by inhibiting the activity of the serine/threonine-specific phosphatase, PP2A [18]. Interestingly, a recent study found that 10% cyclic stretch activates Akt in vascular smooth muscle cells, suggesting that physiologic levels of stretch may also inhibit smooth muscle cell apoptosis [19].

The capacity of cyclic stretch to inhibit vascular EC apoptosis may play an important vasculoprotective and anti-atherogenic role in the circulation. Apoptosis of ECs participates in plaque development by promoting the infiltration of inflammatory cells and lipids into the intima of the vessel wall as well as the proliferation and migration of underlying vascular SMCs. Moreover, the recent finding that a significant percentage of luminal ECs undergoes apoptosis in human atherosclerotic plaques [5] suggests that this process may contribute to plaque erosion and rupture. In addition, apoptotic ECs exhibit marked procoagulant activity and may enhance plaque thrombogenicity following plaque rupture [20]. Thus, the capacity of physiologic cyclic stretch to block EC apoptosis may play a critical role in limiting plaque progression, erosion, and thrombosis.

Interestingly, the effect of cyclic stretch on vascular cell apoptosis is dependent on the magnitude of stretch. Although physiologic levels of cyclic stretch (6–10%) inhibit EC death, a higher level of cyclic stretch (20%) stimulates EC apoptosis. This latter finding complements recent studies in vascular smooth muscle cells where elevated levels of cyclic stretch (15 and 25%) have also been shown to induce apoptosis [21,22]. Under these high levels of repetitive mechanical strain, apoptosis appears to result from oxidative DNA damage and the subsequent activation of the tumor suppressor p53 [23]. High levels of cyclic stretch are encountered in certain pathological conditions, such as hypertension and following the introduction of a venous graft into an artery [24–26]. In this respect, increased rates of vascular cell apoptosis and death have been reported in spontaneously hypertensive animals and in experimental vein grafts [21,26,27]. Thus, the ability of high levels of cyclic stretch to stimulate vascular cell apoptosis may contribute to the pathological remodeling response observed in hypertensive vessels or in vein grafts.

In conclusion, the present study identified cyclic stretch as a critical regulator of EC survival. While physiologic levels of cyclic stretch protect ECs against apoptosis via the activation of the PI3K signaling pathway, higher, potentially pathological levels of mechanical stretch stimulate apoptosis. The abil-

ity of physiologic cyclic stretch to inhibit EC apoptosis may represent a previously unrecognized mechanism by which hemodynamic forces exert a vasoprotective and anti-atherogenic effect in the circulation.

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